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EXPERIMENTAL STUDY

Betulinic acid may modulate autophagy in renal cell carcinoma cells

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ABSTRACT

OBJECTIVES: Betulinic acid is pentacyclic triterpenoid known to exert antitumor effects by modulating many cellular pathways in various human malignancies. However, its modulatory role in autophagy in renal cell carcinoma remains unclear. Here, we observed how betulinic acid affects autophagy in renal cell carcinoma cells.
METHODS: After treating cells with betulinic acid, we determined the gene expression and protein levels of Beclin-1 and ATG-5 by qPCR and ELISA assay to observe its effects on autophagy.
RESULTS: The qPCR results demonstrated that Beclin-1 expression level was low in untreated metastatic renal adenocarcinoma ACHN cells and increased in response to 25 μM and 50 μM betulinic acid treatment. ATG-5 expression level was decreased in primary clear cell renal cell carcinoma CAKI-2 cells treated with 50 μM betulinic acid. In the ELISA assay results, we observed that betulinic acid caused a decrease in Beclin-1 protein level at 25 μM concentration and in ATG-5 protein level at 50 μM concentration in CAKI-2 cells.
CONCLUSION: In current study, it was concluded that the role of autophagy may differ in renal cell carcinoma cells depending on their origin and that the effects of betulinic acid on autophagy in these cells may vary accordingly *(Fig. 4, Ref. 40)*. Text in PDF *www.elis.sk*
KEY WORDS: betulinic acid, autophagy, kidney, cancer, cell.

Introduction

Renal cancers constitute a great majority of cancer types originating from epithelial cells of renal tubule in the kidney. There are many histological subtypes of renal cancer representing many distinct pathological features while the predominant (> 70 %) subtype is referred to as clear cell renal cell carcinoma (ccRCC) (1). In 2020, renal cancer affected over 400,000 people, and led to social and economic burden carried by individuals (2). Although the incidence is higher in people over 65 years of age, it has been observed that the incidence in young adults under 40 years of age has increased in recent years (3). The specification of treatment options substantially depends on disease stage and localization. Fundamentally, when the tumors are localized and in early stage, they are treated with partial or radical nephrectomy (4). The facts that over 30 % of patients experience relapse after successful nephrectomy, and approximately 30 % of renal cancers are metastatic and advanced at diagnosis, have directed the treatment options to chemotherapy and immunotherapy (5). Since the ccRCC is an immunogenic tumor, the tyrosine kinase inhibitors and successful antiangiogenic therapies, were complemented with immune checkpoint inhibitors, which has led to significant advances in the treatment of RCC (6).

Another promising strategy to improve the efficacy of current therapeutics lies in combination therapy of approved targeted drugs or immune checkpoint inhibitors. Despite the great benefits in patients who receive these treatments, urologists face some difficulties in treating these patients. Since RCC has a heterogeneous tumor structure that may contain different mutations, the response to these treatments may vary from patient to patient. These tumors may also have the ability to develop drug resistance which renders administered therapies ineffective and allows tumor cells to survive (7). On the other hand, besides the beneficial combination with certain therapeutics such as lenvatinib plus everolimus and pembrolizumab plus axitinib as compared with a single drug, some other combinations such as bevacizumab plus sunitinib or everolimus can cause substantial side effects ranging from hypertension to pulmonary embolism (8, 9). Given these limitations in the treatment of RCC, there appears to be an urgent need for new drug candidates that can have few side effects, improve response to treatment, and make it relatively curable.

Natural products that have been researched as a potential treatment option in recent years give promising results in cancer treatments as well as in many human diseases. Unlike current

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therapeutic agents, these products should exhibit low toxicity and high bioavailability (10). Betulinic acid is a lupane type pentacyclic triterpenoid which is a substance widely distributed in many diverse plant species (11). Evidence shows that betulinic acid is effective against many human diseases such as HIV, malaria, inflammation, bacterial infection and cancer (12). Particularly its anticancer effect has attracted great interest in new drug research in recent years. The most important feature of betulinic acid's antitumor effect of making it safer to use is that it has selective cytotoxicity on cancer cells without damaging non-cancerous cells (13). Moreover, betulinic acid suppresses many pathways crucial for tumorigenesis such as mitochondrial apoptosis, cell cycle, metastasis, and angiogenesis (14, 15). Although it is known that betulinic acid gives effective results in many types of cancer including renal cancer, its mechanism of action and molecular targets are not yet fully understood.

Autophagy is an evolutionarily highly conserved degradation mechanism in which cellular components such as damaged organelles, misfolded proteins and other cellular macromolecules are destroyed. In other words, autophagy is a lysosomal degradation that removes damaged proteins and organelles to preserve cellular quality and maintains cellular metabolism and survival by providing nutrients to the cell by destruction of cellular components under starvation and other stress conditions (16, 17). This process is regulated by great numbers of autophagy-related genes (ATG). Autophagosome formation, which is the initial step of autophagy, begins with the inactivation of mTOR to form the ULK1 complex which leads to phosphorylation and activation of Beclin-1-Vsp34 complex. The elongation step is regulated by two conjugation complexes called ATG-5-ATG12 and LC3-PE. The formed autophagosome fuses with the lysosome to become an autolysosome, and proteins, organelles or macromolecules are degraded (18, 19). Any disruption in this regulation is closely associated with some human diseases such as obesity, diabetes, neurodegenerative diseases, diabetes and cancer (20). The role of autophagy in cancer is contradictory, such that while it can suppress cancer formation in the early stage, it is known to contribute to progression by providing the necessary nutrients for cancer cells in the advanced stage (21). Moreover, the effects of autophagy in cancer cells also depends on types of cancer. Therefore, elucidating the role of autophagy in some cancer types is important in terms of laying the groundwork for the improvement of new treatment methods.

In this study, we investigated the effect of betulinic acid on autophagy in renal cell carcinoma cells. Thus, we evaluated whether targeting autophagy with betulinic acid in the treatment of renal cancers would bring about a successful result in the future.

Materials and methods

Regents and assays

Betulinic acid was obtained from Sigma Aldrich (St Louis, MO, USA). For experiments, a stock solution was prepared by dissolving it in DMSO (dimethyl sulfoxide) at a final concentration of 100 µM. McCoy's 5A medium, Eagle's Minimum Essential Medium (EMEM), trypsin-EDTA and Fetal Bovine Serum (FBS) were purchased from GIBCO (Green Island, NY, USA). CAKI-2 and ACHN cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA). RNA isolation and cDNA synthesis kits were purchased from Thermo Fisher Scientific (Massachusetts, USA). Beclin-1 and ATG5 ELISA kits were purchased form Abbkine Scientific (California, USA).

Cell culture

ACHN (metastatic renal adenocarcinoma) and CAKI-2 (primary clear cell renal cell carcinoma) cells were cultured with McCoy's 5A medium, and EMEM supplemented with 10 % FBS and 1 % penicillin-streptomycin in 75 cm^3 cell culture flasks. Cells were incubated in humidified incubator with 5 % CO_2 at 37 °C.

WST-1 cell cytotoxicity assay

In our study conducted in our laboratory in previous years, we determined the cytotoxic effect of betulinic acid on renal cell carcinoma cells by WST-1 assay (22). Cells were seeded in 96-well plate at $1x10^4$ cells per well and incubated for 24 h. Following the end of the incubation period, cells were treated with a progressive dosage (1–50 µM) of betulinic acid and incubated for 24 h, 48 h and 72 h. After each incubation period, 10 µl WST-1 solution was added in each well. At the end of the 4-h incubation step, the plate was read at 440 nm using microplate reader.

ELISA assay

Cells were incubated in 75 cm^3 cell culture flasks with determined dosages of betulinic acid for 24 h. Then the treatment cell culture supernatants were used to determine Beclin-1 and ATG-5 protein levels. All experimental steps were performed in accordance with the manufacturer's instructions. Standards and samples diluted with dilution buffer were added in ELISA plates at 50 µl and 10 µl, respectively. At the end of the incubation, after each well had been washed with Wash Buffer, HRP-conjugated antibody was added to all wells except blank wells. Chromogen A and B solutions were added to each well following the incubation and washing steps. A volume of 50 µl of stop solution was added in wells and there was a color change from blue to yellow in each well. ELISA plates were read at 450 nm using microplate reader. Each experiment group and standards were worked in duplicate.

qPCR assay

Cells were seeded in 25 cm^3 cell culture flasks at $2x10^5$ cells in each flask. After the betulinic acid treatment at determined dosages, cells were lysed according to total RNA isolation kit's instructions to perform total RNA isolation. The cDNA synthesis was performed from total RNA samples by High-Capacity cDNA Reverse Transcription Kit. Using cDNA samples, Real-Time Polymerase Chain Reaction (qPCR) was performed to detect Beclin-1 and ATG-5 gene expression levels. TaqMan probe was used, and the reaction conditions were as follows: denaturation at 95 °C for 15 s; annealing at 60 °C for 1m; and elongation at 72 °C for 30 s for 40 cycles. Gene expression levels were calculated as ΔCT, and GAPDH gene was used for normalization of the experiments. Each experiment group was worked in duplicate.

Statistical analysis

Statistical analysis of the data obtained from the experimental results were performed using the SPSS 21.0 package program. The statistical significance limit was accepted as $p < 0.05$. Mann-Whitney U-test was used in the evaluation between groups.

Results

Betulinic acid decreases cell viability in renal cell carcinoma cells

According to the results of our previous study (22), betulinic acid dramatically decreased cell viability in ACHN and CAKI-2 cells. However, betulinic acid did not have a significant effect on cell viability of healthy cells. To apply in other analyses, the effective doses of betulinic acid on renal cell carcinoma cells were determined as 25 µM and 50 µM, and optimal incubation period to 24 h.

Different doses of betulinic acid inhibit Beclin-1 and ATG-5 protein levels

We determined the effect of betulinic acid whose effective doses were chosen as being 25 µM and 50 µM on the autophagy related to proteins Beclin-1 and ATG-5 in renal cell carcinoma cells. According to our observation, betulinic acid caused 34 % decreases in Beclin-1 protein level at a concentration of 25 µM in CAKI-2 cells as compared with untreated cell group. Similarly, in ACHN cells, we observed 11.4 % and 12.2 % decrease in Beclin-1 protein level at concentrations of 25 µM and 50 µM, respectively (Fig. 1). As to ATG-5 protein level, there was no significant change in CAKI-2 cells treated with 25 µM betulinic acid, while a 16.06 % decrease was observed in the cells treated with 50 µM betulinic acid. In the ACHN cells treated with 50 µM betulinic acid, a decrease by 16.04 % was detected (Fig. 2).

Betulinic acid inhibits ATG-5 gene expression while enhancing Beclin-1 gene expression level

To investigate the effect of betulinic acid on autophagy-related genes ATG-5 and Beclin-1 in renal cell carcinoma cells we performed a qPCR assay. The results varied by cell type, such that the Beclin-1 gene expression level was significantly increased in metastatic renal adenocarcinoma ACHN cells treated with 25 µM ($p = 0.01$) and 50 µM ($p = 0.021$) betulinic acid (Fig. 3), while no significant change in ATG5 gene expression level was observed. On the other hand, in primary clear cell renal cell carcinoma CAKI-2 cells treated with 50 µM ($p = 0.021$) betulinic acid, the ATG-5 gene expression level was decreased (Fig. 4), while no significant change in Beclin-1 gene expression level was observed. The results show that betulinic acid may have different effects on Beclin-1 and ATG-5 gene expression levels, depending on whether the cells are primary or metastatic.

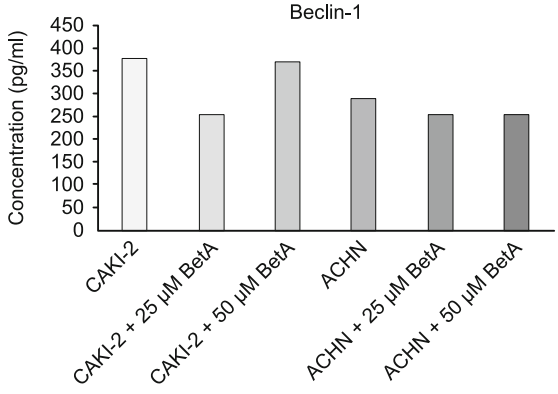

Fig. 1. Betulinic acid caused a significant decrease in Beclin-1 protein level at 25 µM concentrations in CAKI-2 cells. In ACHN cells, a decrease in Beclin-1 protein level at 25 µM and 50 µM concentrations. (BetA = Betulinic acid).

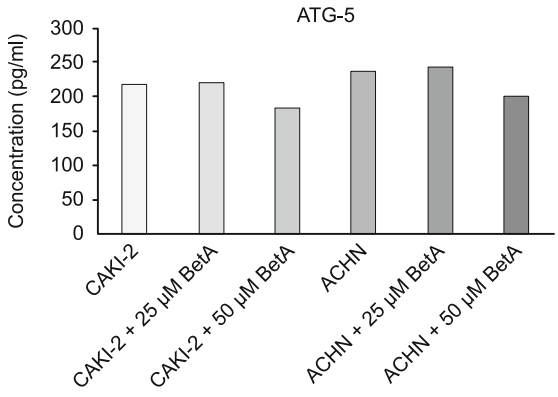

Fig. 2. There was a decrease in ATG-5 protein level in CAKI-2 cells treated with 50 µM betulinic acid. In the ACHN cells treated with 50 µM betulinic acid, a decrease in ATG-5 protein level was detected. (BetA = Betulinic acid).

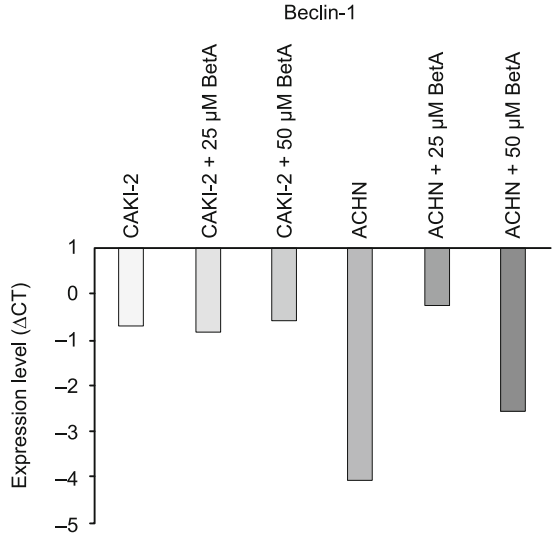

Fig. 3. Betulinic acid enhances Beclin-1 gene expression level and inhibits ATG-5 gene expression level. Beclin-1 gene expression level was decreased in untreated ACHN cells and after the 25 µM ($p = 0.01$) and 50 µM ($p = 0.021$) betulinic acid treatment Beclin-1 gene expression level was significantly increased. (BetA = Betulinic acid).

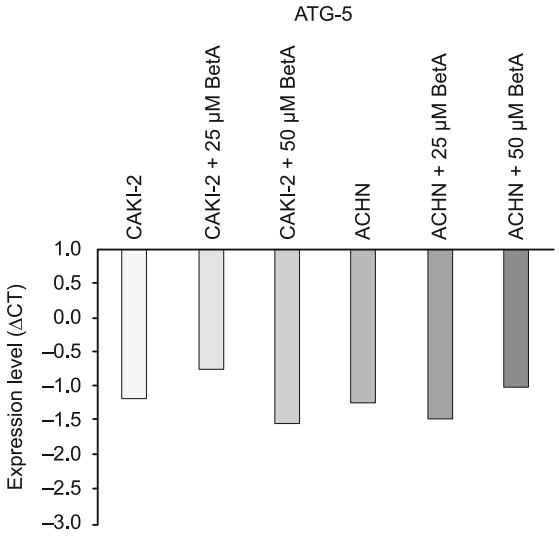

Fig. 4. In CAKI-2 cells treated with 50 µM (p = 0.021) betulinic acid, the ATG5 gene expression level was decreased. (BetA = Betulinic acid).

Discussion

In the treatment of RCC, a significant progress has been made in the treatment options developed for both the early stage and advanced disease which is responsible for a substantial part of RCC patients' mortality. However, the high resistance of RCC to treatments and dangerous side effects complicate the management of treatment and cause the overall survival rate to remain poor (23). In the circumstances, the requirement for the development of novel treatments has led to an increased interest in the use of natural plant-derived compounds with low toxicity and safer use in treatment. In the process of developing natural plant-derived compounds as new therapeutic agents, the efficacy and molecular targets of these compounds should be investigated in detail to constitute a time- and cost-effective strategy (24, 25). In the current study, we researched the role of betulinic acid in autophagy in the treatment of RCC cells. The results suggested that the role of autophagy in RCC may differ according to cell types and thus the effects of betulinic acid on autophagy-related genes may also differ.

Autophagy is a physiological mechanism, in which cellular components are destroyed to maintain cellular homeostasis and metabolic continuity in health, however becomes pathophysiological in some human diseases such as cancer. The role of autophagy in cancer is controversial and depends on the cancer type, stage, and genetic mutations of the tumor (21, 26). Some studies have shown that overexpression of Beclin-1, the central regulator of autophagy, is associated with poor prognosis in stage III colorectal cancer (27), while its low expression in breast cancer as compared to normal tissues may promote tumor progression (28). Considering the beneficial effects of autophagy in healthy tissues and its tumor suppressor or oncogenic role in malignant tissues, it is critical to determine how, in which ways, and in which cancer types, autophagy will be targeted (29).

It is known that the role of autophagy in RCC can be either suppressive or progressive. Autophagy can suppress tumor development by reducing oxidative stress and eliminating dysfunctional proteins in RCC, while supporting tumor progression by protecting against lack of nutrient in the tumor microenvironment and contributing to the drug resistance mechanism, which is one of the most important challenges in RCC treatment (30, 31). Because of this dual role of autophagy in RCC, autophagy inducers such as silibinin, rasfonin and sinomenine and inhibitors such as chloroquine, hydroxychloroquine and 3-methyladenine have been developed (32–34). However, an important point is that while targeting autophagy, attention should be paid not to adversely affect physiological autophagy in healthy tissues and not to cause side effects. In this context, the use of naturally derived compounds known for their low toxicity in targeting autophagy can yield effective results (35). In RCC, natural compounds such as curcumin (36) and resveratrol (37) are being investigated as autophagy modulators.

Betulinic acid, a pentacyclic triterpenoid, is a naturally occurring bioactive compound with anticancer properties. Although the effects of betulinic acid on many cellular pathways are known, it is unclear whether its effect on autophagy is inhibitory or activating. Yang et al showed that in multiple myeloma cell lines, betulinic acid inhibits autophagic flux by causing downregulation of the main autophagy activator, Beclin-1 and accumulation of LC3-II (38). On the other hand, in another study, it was shown that betulinic acid induces autophagy by increasing autophagosome formation in colorectal cancer cells and by increasing the expression of Beclin-1 ATG12, ATG7 and ATG5 genes. In addition, it was observed that betulinic acid-induced cellular apoptosis increased and proliferation decreased because of autophagy blockade with autophagy inhibitors. Therefore, it has been reported that autophagy is activated in response to betulinic acid treatment and plays a protective role against treatment in colorectal cancer cells (39). These studies show that the modulatory role of betulinic acid on autophagy may vary according to cancer type, and it remains unclear how this role affects cancer cells.

In our study, we observed the change in Beclin-1 and ATG-5 expression and protein levels caused by betulinic acid treatment to investigate the effect of betulinic acid on autophagy in renal cancer cells. We found that Beclin-1 expression level was low in untreated metastatic adenocarcinoma ACHN cells and increased in response to 25 uM and 50 uM betulinic acid treatment. Deng et al. showed that autophagy-related genes and autophagy level were lower in advanced or metastatic renal clear cell carcinoma than in localized tumors (40). Accordingly, the lower Beclin-1 level in untreated ACHN cells as compared to CAKI-2 cells in our results may be due to the metastatic character of ACHN cells and autophagy may be induced protectively against betulinic acid treatment. On the other hand, 50 µM betulinic acid treatment significantly reduced ATG-5 gene expression in primary clear cell renal carcinoma CAKI-2 cells. In line with this result, betulinic acid treatment at the same dose also decreased the ATG-5 protein level.

Taken together, according to these results, the role of betulinic acid on autophagy in renal cell carcinoma cells seems to vary depending on the origin of the cells. Our results show that this study is a preliminarily study set forth that autophagy can be targeted

with betulinic acid in renal cancers. Further studies are needed to determine the role of betulinic acid on autophagy in renal cancers and to determine its molecular targets and whether it can be used effectively in treatment.

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